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September 1975

FIELD IONIZATION MASS SPECTROMETRIC
RAPID DIAGNOSIS OF INFECTIOUS DISEASES

Supported by

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EXECUTIVE SUMMARY

↙ This report describes the second phase of a research program aimed at the utilization of multicomponent analysis by field ionization mass spectrometry for diagnosis of infectious diseases. During this phase it has been demonstrated that infectious hepatitis can be diagnosed with a high degree of confidence through analysis of the molecular weight profile of the acidic metabolites in urine. New sample preparation techniques, instrumentation, and computerized statistical analysis have been developed during this phase of the program. ↗

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I INTRODUCTION

This is the second annual report on a program on "Field Ionization Mass Spectrometric Rapid Diagnosis of Infectious Diseases" conducted under Contract DAMD17-74-C-4047 with the U.S. Army Medical Research and Development Command. This report, which covers the period December 1, 1974 through October 15, 1975, is being submitted at this time to support our proposal No. PYU 75-271, dated September 29, 1975, for continuation of this paper. Our objective in this paper is to develop a methodology for the rapid diagnosis of infectious diseases based on nonfragmenting mass spectrometry. The experimental approach is based on a novel methodology developed at SRI using new types of multipoint field ionization sources. This methodology allows the detection of specific metabolic aberration that may occur in the host as a result of an infectious process, as well as the identification of microorganisms by their chemical constitution or by their characteristic metabolic products.

The capability of making a rapid and reliable diagnosis of infectious diseases at an early stage and at low cost would be of especially great value to the military where large numbers of soldiers are often stationed in confined areas and their continuing health is crucial to carrying out their objectives. Early and reliable diagnosis of an infectious disease could prevent the spread of disease to large groups of soldiers and civilians on the post.

During the first nine months of the second, ongoing phase of this project, we have developed reproducible methods for the mass spectrometric determination of carboxylic acids in urine, have then worked out faster and more reliable procedures for the separation of carboxylic acids and of general basic constituents from urine, and have made progress

in the development of separation procedures of primary amines from urine. We have also developed computer techniques for the analysis of multi-component spectra and for the pattern recognition analysis of metabolites to identify aberrations associated with pathological conditions. We have analyzed adequately large groups of urine samples from patients having infectious hepatitis, have compared these analyses with urine of normal subjects, and have established characteristic differences in the molecular weight profiles of carboxylic acids of the two groups.

II SUMMARY OF ACCOMPLISHMENTS

The objective of the proposed program of research is to answer the following questions:

- Do certain or most infectious diseases exhibit characteristic concentration patterns of metabolites in plasma or urine?
- To what extent are the changes in the chemical constitution of these biological fluids indicative of the severity of infection?
- To what degree can the chemical aberrations in urine be used as indicators for recovery, and to what degree can they be useful for the identification of post-infection carriers?
- Do carriers, who do not exhibit any clinical symptoms of infection, produce any significant characteristic metabolites?
- Can microorganisms in minute quantities be identified through a characteristic nonpolymeric biochemical fingerprint?
- Can microorganisms be identified, through their characteristic metabolites released into controlled artificial media, within one to three hours of incubation?

To answer these questions, we envisage an extensive program of research that will take five years or more. All the basic instrumentation necessary for the feasibility phases is currently available and operative. We are adding a fragmentation chamber setup and a minicomputer to our systems. The advanced phases of the proposed research program will thus benefit from the augmented capabilities of our analytical systems.

Within two to three years, multipoint field ionization sources as well as the general SRI-developed rapid integrated multiscan methodology will become available in other research centers so that efforts would proceed in parallel to implement the proposed

diagnostic technique. We will also be ready as part of a parallel contract to develop an appropriate mass spectrometric system for the U.S. Army Medical Research and Development Command.

In our original proposal, we proposed to explore the feasibility of three fundamental assumptions behind this program.

- That certain infectious diseases result in characteristic biochemical manifestations in plasma or urine.
- That microorganisms have characteristic nonpolymeric chemical fingerprints.
- That microorganisms have characteristic nonpolymeric fingerprints of metabolites.

To explore these assumptions, we proposed to test different urine extracts from normal subjects, from patients with established infectious diseases, and from the same patients following recovery. Chemical fingerprints were to be determined in different extracts of the urine, and if characteristic aberrations were observed, attempts were to be made to select methods of fractionation that would enhance the demonstration of these changes.

Next we proposed to examine chemical fingerprints of the nonpolymeric constituents of different separated microorganisms following extraction. We also planned to examine artificial growth media for changes in chemical composition following short periods of incubation with different microorganisms.

In the second year, our objectives were to:

- I. Investigate changes in amino acid composition in the urine of patients suffering from infectious diseases, particularly, infectious hepatitis and mononucleosis.

II. Carry out the same type of investigation on α -keto and hydroxy-phenyl carboxylic acids in urine with special emphasis on phenolic acids.

III. With the aid of a computer, establish the feasibility of distinguishing between closely related bacterial strains by their biochemical fingerprints.

We have met the second objective of the program and were able to demonstrate significant differences between the molecular weight profiles of carboxylic acids between normals and patients with infectious hepatitis. These results corroborate the assumptions made in last year's proposal. Instead of amino acid analysis we are now examining the molecular weight profile of the neutral extracts of urine, and the preliminary results indicate significant differences, as in the case of carboxylic acids. This phase will be completed before the end of the second year of the program. We have deferred the experimentation on microorganisms (Objective III). The instrumental and computational techniques developed during the ongoing second phase will facilitate the rapid implementation of this aspect of the program in the proposed third phase.

III TECHNICAL BACKGROUND

This section includes updated elements of the first annual report as well as the results of the experimental effort during the first ten months of the second phase of the project.

A. Chemical Diagnosis Through Multicomponent Analysis

Every disease, whether infectious, endocrine, allergic, or even neurological, is accompanied by physiological changes that are reflected in changes in the chemical composition of the plasma. The chemical composition of the plasma is a function of cellular metabolisms in the different organs. The physiological state of the organism is a manifestation of the physiological states of the individual cells in the separate organs. Changes in the chemical composition of the plasma may be classified in three major groups:

- (1) Periodic or progressive changes of cellular metabolic states within the range of normal physiology (these may be extensive changes as manifested in pregnancy or as a result of maturation or aging of the organism).
- (2) Aberrations in physiological behavior that are not sources of discomfort and are not correlated to any known disease.
- (3) Significant aberrations in the chemical composition of the plasma that are directly correlated with pathological states.

Only the last type of aberrations has clinical diagnostic value.

Once observed and used for diagnosis, a pathological chemical aberration can also be used as an indicator during the therapeutic process to establish the efficiency of treatment and the completeness of recovery.

Moreover, where a specific disease is suspected, and its onset must be determined as soon as possible (such as during an incubation period following the subject's exposure to a known pathogen), the follow-up of the chemical composition of plasma and early detection of specific aberrations are of paramount clinical value, facilitating early and effective "preventive" treatment. Specific metabolic changes associated with infectious diseases will be discussed in subsection B.

Mass spectrometric techniques can be applied best to the assay of the nonpolymeric minor constituents of plasma. Classic analytical chemistry determines quantitatively one constituent at a time. When applied to clinical diagnosis of metabolic disorders, from hormone deficiencies to potential biological tracers of cancer, the assay of a single constituent is used as a diagnostic parameter. This diagnosis can in some cases be corroborated by a second or third single parameter. However, any disorder may be associated with a large number of simultaneous aberrations in the chemical profile of the plasma. For example, thyroid disorders may be differentially diagnosed, not only by the concentrations of nonprotein-bound thyroxine and tri-iodothyronine, but also by a simultaneous upset in the equilibrium of nutrients in the plasma, especially those contributing to protein synthesis. Such a change in the concentration profile of nutrients in the plasma may be far more valuable for diagnosis of thyrotoxicosis than measurement of the basic metabolic rate. Neoplastic cells exhibit quantitative changes in a number of enzymatic pathways, resulting in a possible diagnostic pattern. Although the changes in concentration of individual constituents may be biologically or even methodologically insignificant, the change in pattern, which presents the simultaneous change in a large number of constituents, is expected to be of much higher significance.

Many hundreds and probably many thousands of minor constituents exist, each one of possible diagnostic significance, and it is highly desirable to determine as many of them as possible. Of all analytical methods in use, mass spectrometry is capable of determining the most constituents in the shortest time. Modern rapid scanning nonfragmenting mass spectrometry can determine hundreds of constituents in a few minutes.

Although we are interested in the chemical composition of plasma, the use of urine as the medium for routine investigation has several advantages: (1) Urine is almost free from biopolymers (unless an impaired kidney function exists, which can readily be determined). This is a significant advantage as it alleviates the complications due to protein binding of various nutrients and metabolites. A meaningful analysis of nonpolymeric constituents in plasma might require denaturation of the proteins, which might still entrap some of the protein-bound monomers. Dialysis, column chromatography, or solvent extraction are other alternatives that might complicate the analytical procedure. (2) Since the resorption process in the kidney does not act effectively on many metabolites, urine contains higher concentrations of such metabolites than does the plasma. (3) The collection of urine is noninvasive; thus, many samples may be obtained from the same subject with minimum inconvenience.

To extend the scope of "chemical diagnosis" and make it a more universal diagnostic tool, we need an analytical technique that fulfills the following requirements:

- (1) It should be capable of quantitative analysis of many identified nonpolymeric constituents of plasma or urine in the concentration range 10^{-3} to 10^{-9} M.
- (2) Speed and cost per analysis should be adequate to allow the screening of many hundreds of samples to establish valid correlations between chemical composition and disease.

Modern mass spectrometry, particularly, nonfragmenting field ionization mass spectrometry, fulfills the above requirements of sensitivity, quantitateness, specificity, speed, and cost per analysis.

B. Chemical Diagnosis of Infectious Diseases

The classic diagnosis of infectious diseases is through identification of the pathogen, e.g., virus, bacteria, fungus. This identification requires either isolating the pathogen and then identifying it or identifying it through a specific serological immune reaction. Relatively little information is available, however, on general systemic biochemical aberrations associated with infectious diseases. Most of the cases where biochemical changes have been observed involved biochemical changes in specific infected organs, such as the liver or the kidneys.

The biochemical changes associated with infections may be divided into two categories: (1) changes in plasma protein concentrations that include increase of immunoglobulin concentration or the release of enzymes from viable or degraded cells, and (2) changes in nonbiopolymeric constituents of the plasma, such as amino acids and their metabolites, carbohydrates and lipids, which may be the result of altered metabolism in infected cells, release of metabolites from degraded cells, or enhanced enzymatic activity in the plasma (generally the result of enhanced release of enzymes into the plasma). Further, certain microorganisms release characteristic endogenous enzymes that, unlike endo- or exo-toxins, do not exhibit dramatic pathological effects, but may produce unique metabolic products. For example, it was reported that virulent strains of F. Francisella release citrulline uridase.^{1*} Mixo-

* References are listed on page 51.

viruses produce an enzyme that releases neuraminic acid from glycoproteins during their attachment to cell membranes. Elevated concentrations of neuraminic acid may possibly be detected in the urine of mumps patients, as this acid is likely to have a high kidney clearance.

Although our objective in this program is to identify changes in the concentration of nonpolymeric constituents of plasma, it is of definite interest to us to consider known pathological changes in the concentrations of certain enzymes because these changes may result in corresponding changes in the concentration of their low molecular weight substrates or of their nonpolymeric products.

Systemic metabolic changes have been reported in a number of metabolic systems. For instance, hyperglycemia was reported in bacterial infections (meningococcus meningitis,² tularemia,³ pneumococcal pneumonia,⁴ gram negative septicemia)⁵ and in viral infections (sandfly fever).⁶ Aberrations in lipid metabolism have been observed in gram positive and gram negative bacteremias,^{7,8} including salmonella infections, pneumonias, and in viral infections.⁹ The effect of salmonella endotoxin, studied on lipid metabolism in monkeys,¹⁰ suggests a possible mechanism for the changes in lipid concentration associated with infectious diseases.

Aberrations in amino acid concentrations in plasma or urine were reported in the cases of bacterial infections¹¹ including tularemia,^{12,13} typhoid fever,¹⁴ pneumococcal pneumonia,^{15,16} rickettsial disease (Q fever),¹² and in viral infections including sandfly fever,^{12,17} yellow fever vaccine disease,¹⁸ and varicella.¹⁹ No detailed analysis on the changes in the relative concentrations of many individual amino acids was carried out in any of these studies. Characteristic changes in the total profile of amino acids in urine and plasma have been

demonstrated in ten different types of hepatic disorders.²⁰ Amino acid-uria with a characteristic amino acid profile has been again demonstrated in infectious hepatitis.^{21,22}

Similar characteristic changes in the amino acid profiles may very well be found in various infectious diseases. Conceivably, many of the changes in amino acid metabolism in infectious diseases are mediated by the liver, and an infectious liver disease should therefore be considered a generalized disease.²³

Another mechanism explaining aberrations in excretion of amino acids may involve pathological changes in the kidney induced directly or indirectly by the pathogen. These changes have been demonstrated in cases of viral hepatitis,^{24,25} infectious mononucleosis,²⁶ and pneumonia.²⁷ Since the relation between the relative concentrations of amino acids in plasma and urine is fairly complex,²⁸ the interpretation of aberrations in the profile of amino acids in urine deserves careful consideration.

Another group of nonpolymeric metabolites worth considering from the diagnostic standpoint are the α -keto and α -hydroxy carboxylic acids formed by the corresponding transaminases and hydrogenases. The concentrations of these analogs of pyruvic and lactic acids have been reported to be increased in the presence of viral hepatitis²⁹ as well as in other liver disorders.³⁰ This increase is to be expected in view of the well-known increase in transaminase activity under these conditions.³¹⁻³⁵ Although the best known transaminases are the glutamic-oxaloacetic and glutamic-pyruvic transaminases (probably because of the ease of their assay), much more interesting from the diagnostic standpoint are enzymes producing other keto and hydroxy acids that are not involved in the Krebs cycle. More than ten phenolic carboxylic acids have been identified in human urine,³⁶ most of them formed by

transamination from phenylalanine, tyrosine, tryptophan, and their analogs.³⁷ The presence of significantly increased concentration of these acids and especially, p-hydroxyphenylacetic acid in the urine of hepatitis patients²⁹ is thus not surprising. The renal threshold for these acids is very low;³⁸ thus they may serve as better diagnostic indicators than amino acids or some of the aliphatic keto or hydroxy acids. The many times higher than normal concentrations of 2,5-dihydroxyphenyl pyruvic acid found in children with acute rheumatic fever,³⁹ which may be correlated with the abnormally high concentrations of tyrosine observed in rheumatic arthritis,⁴⁰ is another indication that these acids might have a broad diagnostic value. Because significantly elevated transaminase activity persists even during the postconvalescent period of infectious hepatitis,⁴¹ the examination of hydroxyaromatic acid levels in urine may be a useful tool for identifying carriers of this infectious disease.

Using α -keto and hydroxy carboxylic acids as diagnostic indicators, one must remember that the kidney contains amine oxidases, which produce α -keto acids plus ammonia by an independent mechanism.⁴² Reduction or enhancement of this metabolic pathway may result in changes in the concentrations of certain keto carboxylic acids and in the ratio of α -keto to α -hydroxy acids.

In summary, it seems that there is sufficient reason to believe that the availability of broad spectrum profiles of certain families of metabolites will lead to the demonstration of metabolic changes characteristic of infectious diseases in general or more probably of specific infectious diseases or groups of diseases.

In the first phases of this program, we decided to concentrate on the analysis of urine for carboxylic acids and amines, which are the

deamination, transamination, or decarboxylation products of amino acids. The literature reviewed here indicates a strong likelihood that the concentrations of these metabolites in the plasma and urine will have diagnostic value, helping in the prevention and treatment of certain infectious diseases. Because infectious hepatitis may produce significant aberrations in the concentration profiles of these metabolites, and because this disease is expected to be of interest to the U.S. Army Medical R&D Command, we chose the study of this disease as one of the first objectives of our program.

C. Field Ionization Mass Spectrometry

Nonfragmenting ionization of organic molecules, which is facilitated by field ionization, opens up exciting new possibilities in analytical chemistry in general⁴³ and in clinical chemistry in particular. Field ionization mass spectrometry allows fast quantitative analysis of constituents of complex multicomponent mixtures.⁴⁴ Combined with isotope dilution techniques, it also makes possible the quantitative determination of individual compounds down to 10^{-15} mole and possibly below.⁴⁵ In addition, this unique method of ionization, which does not involve any isotopic scrambling, facilitates the use of nonradioactive multilabeled molecules as tracers in biological systems with a sensitivity matching and occasionally exceeding that of radioisotopic tracers.⁴⁵

The polarization of molecules in a strong electrostatic field (of the order of 10^8 V/cm) may result in the transfer of an electron to the cathode by tunneling. This electron transfer, which takes place at energy expenditures lower than most chemical bonding energies, results in ionization that is not associated with cleavage of chemical bonds.

Field ionization, unlike electron impact or most collisional charge transfer and atom transfer reactions or photolytic ionization processes, produces molecular (parent) ions almost exclusively. Figure 1 includes examples of field ionization spectra of a number of metabolites. The satellite peaks observed in these spectra are those of impurities and not the result of ionization-induced fragmentation. The limited number of cases where positively charged fragments rather than molecular ions are formed by field ionization generally involve ions that are thermodynamically unstable even at their ground states, e.g., $\text{C}(\text{CH}_3)_4^+$ or CCl_4^+ . The efficiency of field ionization is a function of the ionization potential and the polarizability of the ionized species. As the ionization potentials of most organic molecules are within the narrow range 8 to 12 eV, it is easy to avoid excessively high fields that might produce excited ions, and therefore, possible fragmentation.

Field ionization is facilitated by the high field gradient that can be produced at surfaces with very high curvatures. A cathode with a radius of curvature of about 0.1μ and an anode at a distance of 25μ require less than 1000V to produce field ionization. Such a configuration is readily attainable in a reproducible manner by the appropriate technology. SRI has developed the technology to produce arrays of hundreds of microcones on a porous substrate; these arrays constitute the crucial element in our present ionization sources.⁴⁶

Figure 2 presents an array of microcones mounted on a screen. Figure 3 depicts an advanced version of our sources in which the temperature of the sample can be controlled independently of the temperature of the source.⁴⁷ The temperature of the source is maintained constant and higher than the maximum temperature the sample is subjected to; this prevents memory effects and results in more controlled ionization conditions.

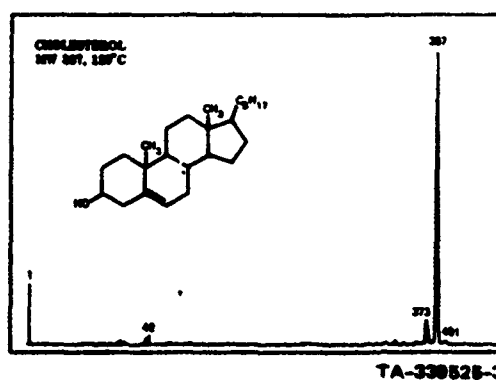
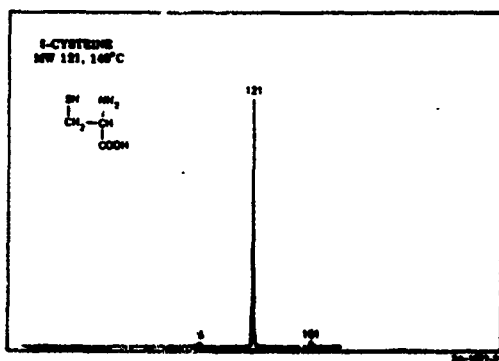
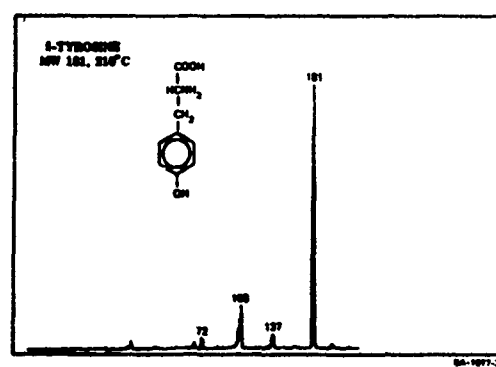
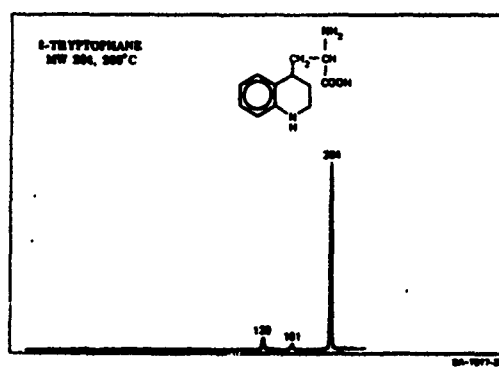
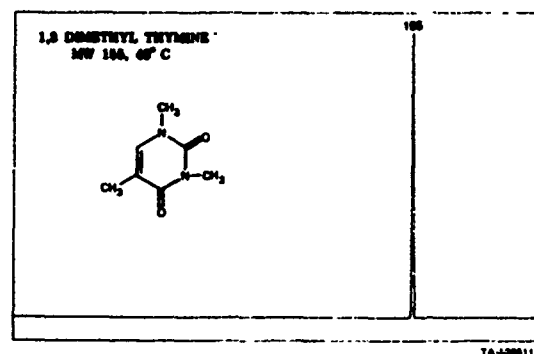
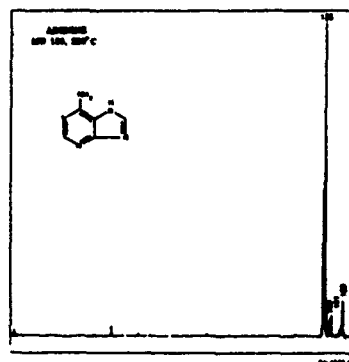
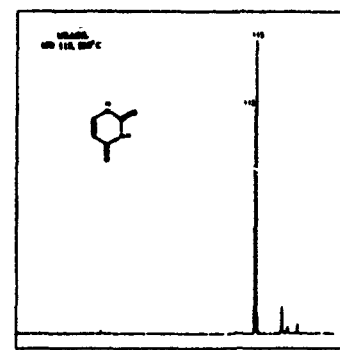
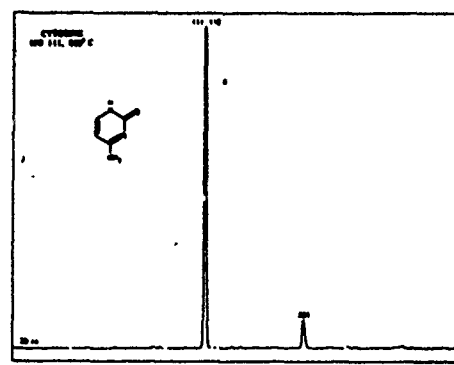
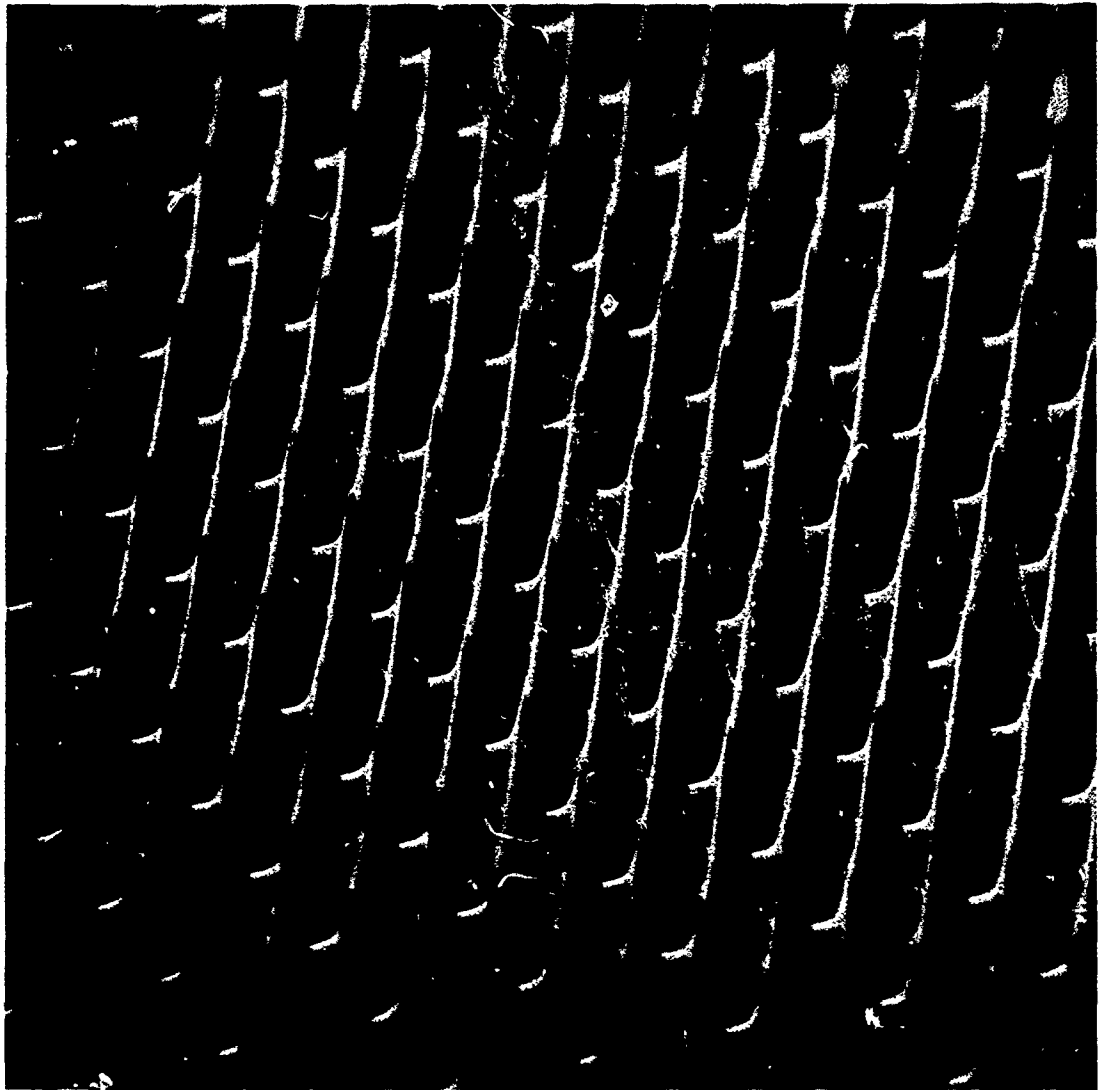
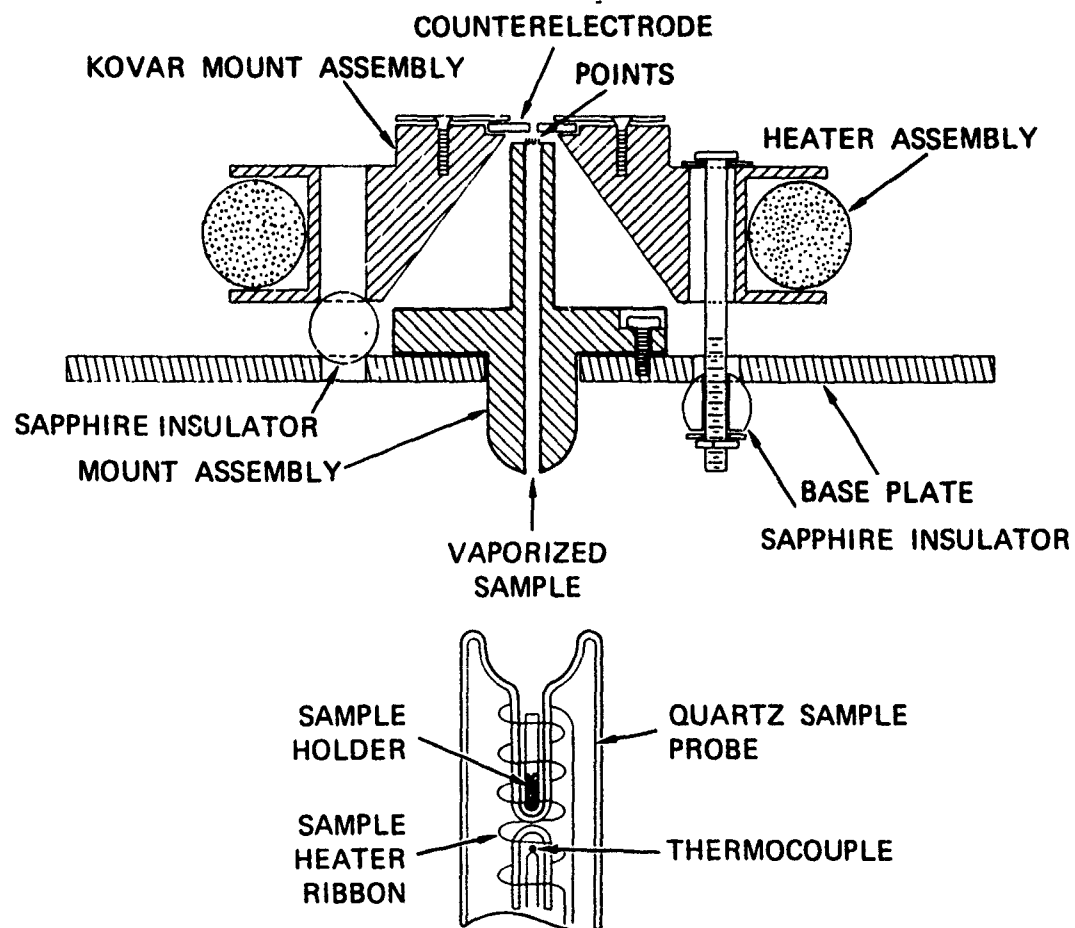


FIGURE 1 NONFRAGMENTED FIELD IONIZATION SPECTRA OF SEVERAL METABOLITES



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FIGURE 2 SCANNING ELECTRON MICROGRAPH OF MULTIPOINT STRUCTURE



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FIGURE 3 SCHEMATIC CROSS SECTION OF MULTIPOINT IONIZER SHOWING SOLID SAMPLE PROBE

At present, we are testing a new source structure with a significantly lower heat capacity which has blackened metal surfaces to enhance radiative heat losses. This new source can cycle between room temperature and 200°C in less than 10 minutes compared with over 45 minutes for the old source. This feature will allow the analysis of 4 to 5 samples per hour, compared with one per hour today.

The ionization efficiency of our screen-mounted ionization sources is 5×10^{-4} or higher,⁴⁸ which is comparable to that of advanced electron impact sources. However, owing to the relatively large area of our ionization source (about 2 mm^2), the high energy of the ions produced, and the divergence, less than 10^{-3} of the ions produced are detected after mass separation.

The overall efficiency of the present generation of field ionization mass spectrometers is about 2×10^{-7} for instruments with a magnetic sector or a Wien ion velocity filter, and about 10^{-9} for the Extra-nuclear Model 270-9 quadrupole mass analyzer, when each of these instruments reaches a resolution of 800-1000. This is not the ultimate overall sensitivity attainable and we are working on improving it.

However, even this relatively low sensitivity allows the determination with 10% precision of as little as 10^{-12} mole with the quadrupole analyzer (when the resolution is lowered to about 200) and as little as 10^{-15} mole with the magnetic sector or the Wien filter analyzers. Even today we are not sensitivity limited in most applications to be discussed. In the analysis of multicomponent mixtures, such as biological fluids, we are not sample limited. Thus 100 μg of material, which is an adequate sample, allows us to determine with a 10% precision components that constitute 10^{-4} of the total mixture (when we scan over a range of 200 amu) even with the quadrupole analyzer.

Using the magnetic sector, the sensitivity increases and allows the determination of constituents present at the ppm level. Using the abundance ratio mode and introducing a 100-ng sample of, say, thymine, we can now determine as little as 10^{-14} mole or 1 pg of thymine with a 3% precision.

D. Analysis of Complex Multicomponent Mixtures

As stated above, field ionization, thanks to its nonfragmenting nature, allows the analysis of multicomponent mixtures. Each peak in a mass spectrum of such a mixture is a measure of the abundance of a single constituent or of the sum of all the isomers and isobars having the same gross molecular weight. However, the composition of the vapor phase over a multicomponent mixture changes as it evaporates into the ionization source, leaving the less volatile constituents behind. Reproducible quantitative analysis of multicomponent mixtures can be achieved in two ways: (1) by flash-evaporating the whole mixture, making the composition of the vapor phase equal to that of the sample; and (2) by integrating many rapidly scanned mass spectra of the mixture, obtained while it evaporates, into a composite mass spectrum that represents quantitatively the composition of the sample. The requirements in this case are that the rate of each individual scan should be fast compared with the rate of evaporation of the sample, and that the scanning be continued until complete exhaustion of the sample.

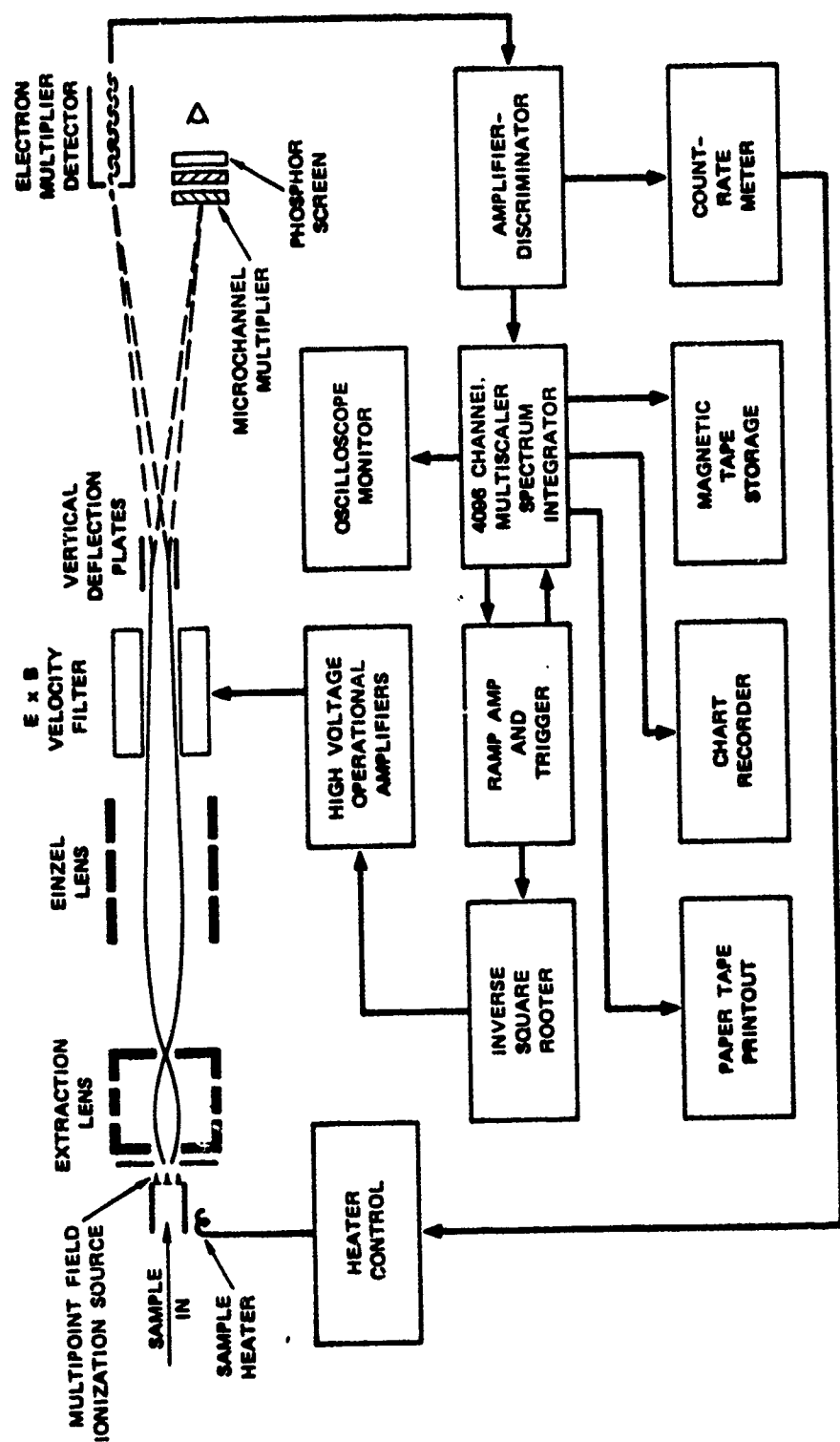
We have adapted both the E x B Colutron ion velocity filter and the Extranuclear quadrupole for a multiscanning mode of operation.⁴⁹ Figure 4 is a schematic description of the first instrument. The ramp voltage taken from the time base of the multiscaler (Nuclear Data ND-2400, 4000-channel analyzer) is amplified after conversion to a linear mass time dependence to produce a changing voltage on the electrostatic plates

of the velocity filter.⁴⁴ Figure 5 is a multiscan of a crude oil sample produced on this instrument. The resolution of 700 obtained here is an important achievement considering that this is a composite spectrogram obtained from hundreds of individual scans.

Figure 6 is a schematic presentation of the multiscan arrangement of the quadrupole instrument. The major advantage here is the linear mass-time dependence of the quadrupole, which alleviates the necessity for a conversion circuit. The overall ion transmittance efficiency on this system is lower, however. Figure 7 is a multiscan of a coal liquefaction product made with the quadrupole. The quality of this spectrum is comparable with that produced using the velocity filter. We are now adapting a magnetic sector mass spectrometer to multiscan capability using magnetic mass scanning.

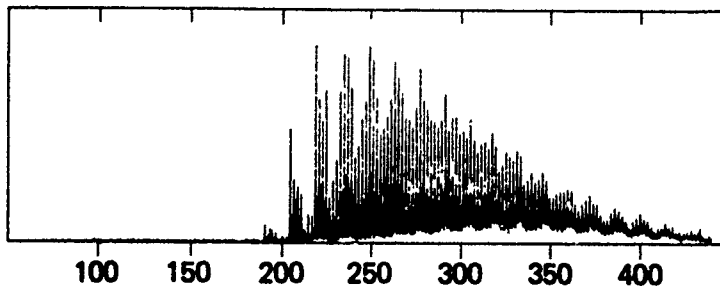
The beam divergence from a field ionization source is large, and it increases with the points-to-counterelectrode potential. Consequently, maximum sensitivity for a given field ionization instrument will be produced by the maximum allowable ion energy and the minimum allowable ionization field. Operation with ionizing potential <1000V eliminates the disturbing effect of superexcited molecules which are field ionized in the quadrupole.⁵⁰ Quadrupoles require ions at a few eV, so the ion transmission is limited in this case. In the best arrangement, the ionizer is placed as close as possible to the quadrupole entrance to minimize losses due to loss of brightness of the divergent beam which falls off approximately as $1/r^2$. These improvements in the interfacing of the field ionization source with the quadrupole allowed us to carry out all the measurements on the urine samples with a quadrupole mass spectrometer.

The quantitative assay measurement of multicomponent mixtures has numerous biomedical applications, from relatively simple applications, such as determining the relative quantities of purines and pyrimidines

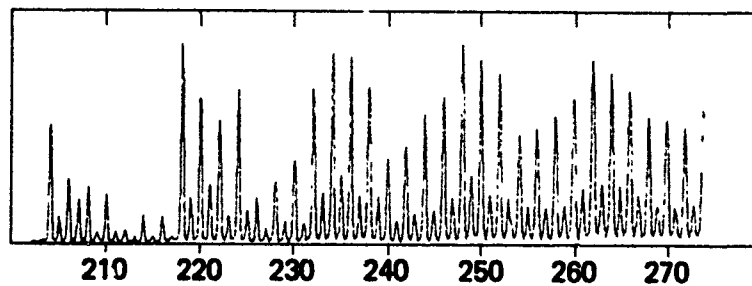


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FIGURE 4 SCHEMATIC OF FIELD IONIZATION FINGERPRINT APPARATUS



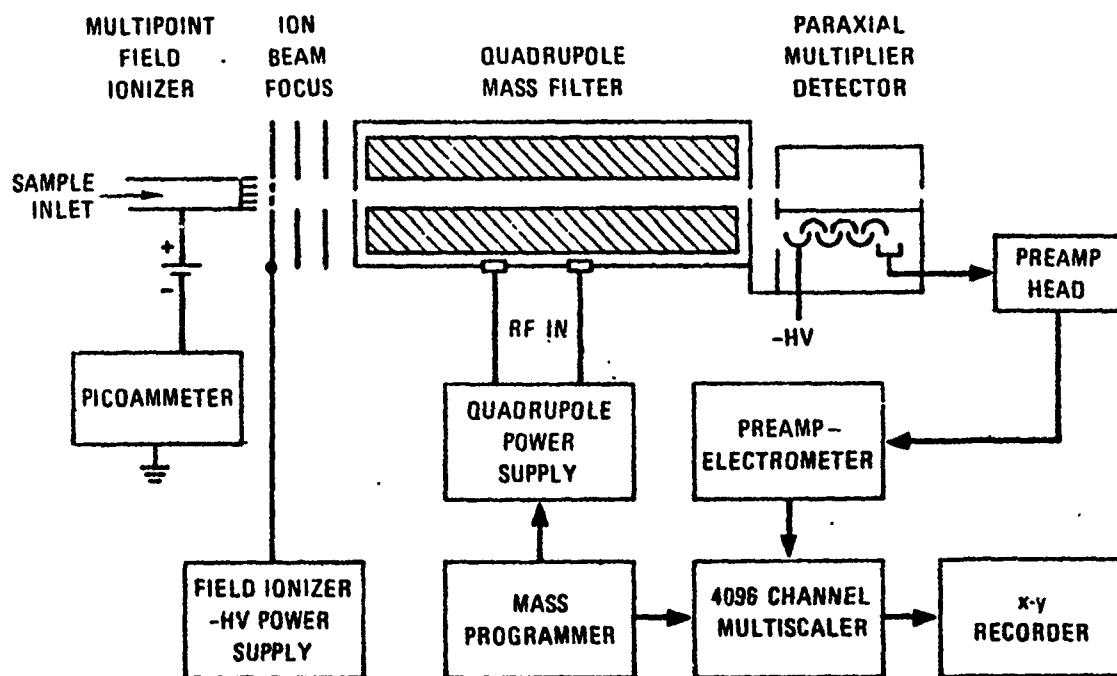
(a) INTEGRATED MASS SPECTRUM OF M.D-CUT QUIRIQUIRE VENEZUELA
CRUDE OIL OVER WIDE MASS RANGE



(b) EXPANDED SECTION

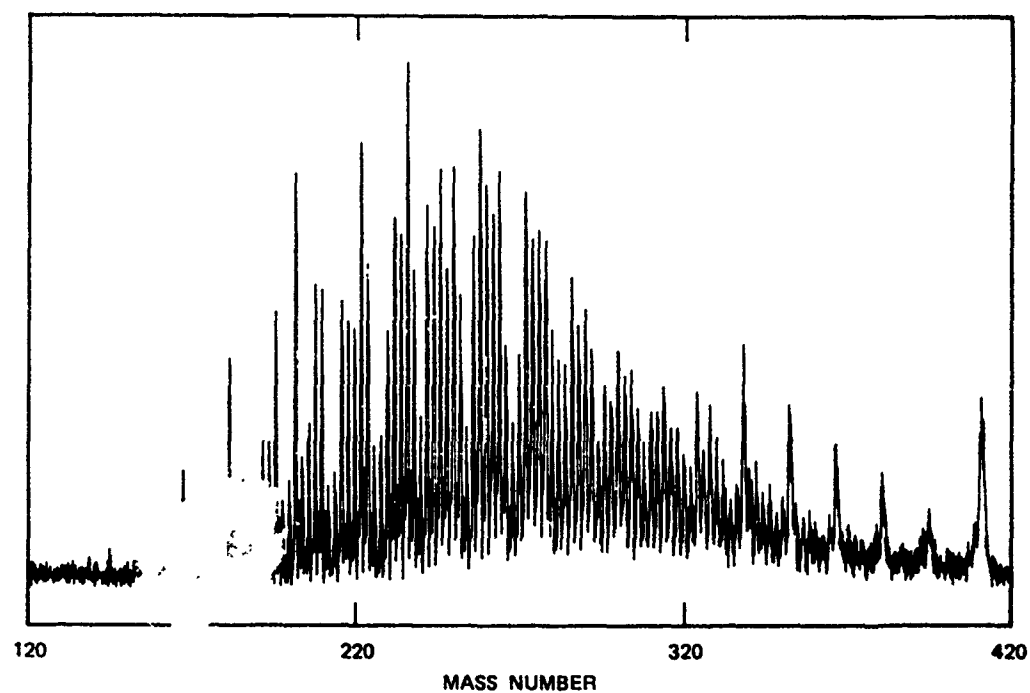
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FIGURE 5 INTEGRATED MASS SPECTRUM OF MID-CUT
QUIRIQUIRE VENEZUELA CRUDE OIL OVER
WIDE MASS RANGE



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FIGURE 6 SCHEMATIC OF FIELD IONIZATION SOURCE, QUADRUPOLE SYSTEM



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FIGURE 7 FIELD IONIZATION INTEGRATED SPECTRUM OF ELUTED FRACTION
OF NO. 1A FROM ARCO H-COAL LIQUID

in nucleic acids, to analysis of complex mixtures, such as urine extracts. Examples of such spectra were included in the first annual report of this project.

The interpretation of a multiscan spectrum of a multicomponent mixture requires somewhat sophisticated computer techniques to extract maximum useful information. We must distinguish between significant differences in spectra and trivial differences caused by instrumental and statistical factors or due to irreproducibility of the pretreatment procedure. This distinction is important when we are interested in establishing new metabolic disorders related to certain diseases and even more so when metabolic profiles are to be used to predict the onset of disorders. The statistical computerized procedure to establish significant differences between normal and pathological samples is presented in Section F.

E. Multicomponent Mass Spectrometric Analysis of Urine

Multicomponent analysis of urine using gas-liquid chromatography (glc) has been used to demonstrate the differences between normal and diabetic patients,⁵¹⁻⁵³ as well as to diagnose inborn errors of metabolism.⁵⁴ Glc can also be used for amino acid analysis,^{55,56} and in clinical chemistry, glc was applied to the specific case of phenyl alanine in urine.⁵⁷ In combination with conventional electron impact mass spectrometry, glc has been used to determine urinary acid profiles in normal subjects⁵⁸ and in subjects under the effect of alcohol.⁵⁹ This work follows previous studies using the same techniques.⁶⁰⁻⁶² A more recent study has demonstrated significant differences in urine metabolite concentration patterns in the case of multiple sclerosis.⁶³

In the first annual report on this project, we discussed the advantages of nonfragmenting field ionization mass spectrometry for

multicomponent analysis of metabolites and demonstrated the applicability of the technique to the analysis of urine extracts (pH = 9) in ethyl acetate. Figure 8 demonstrates again the reproducibility of the technique.

The amount of information attainable by observing all possible metabolites in a urine extract is too large and the potential interference by isomers and isobars too extensive to allow a meaningful analysis of aberrations caused by a specific metabolic factor. We preferred, therefore, to limit ourselves to the analysis of two families of compounds, namely, amines and carboxylic acids. These two families of compounds seem at present most promising for the biochemical diagnosis of infectious diseases.

The separation procedure preceding mass spectrometric analysis of carboxylic acids used in the early stages of our work was a modification of that used by Armstrong et al.³⁶ Urine pretreated with urease at pH = 7 was extracted at neutral pH (phosphate buffer) to remove lipids with diethyl ether. The aqueous phase was then acidified with HCl to pH = 1, saturated with NaCl, and extracted with ethyl acetate or ethyl formate. The extraction was repeated three times, and the organic phases were pooled together. The organic solution was then treated with saturated aqueous NaHCO_3 , which removed the acids into the aqueous phase. This phase was then separated, reacidified with HCl at 0°C , saturated with NaCl, and extracted three times with ethyl acetate. The organic phase was then dried over Na_2SO_4 , separated and evaporated to dryness with a rotary vacuum evaporator at room temperature at 25 mm Hg. The residue was then dissolved in a minimum volume of hexafluoroisopropanol and transferred in solution into the glass capillary container used for the mass spectrometric analysis. The solvent was then removed by a stream of dry nitrogen at 40°C . The mass spectra of the carboxylic acids in the range 71 to 214 amu are presented in Figure 9. We have been able to identify practically all carboxylic acids previously identified by

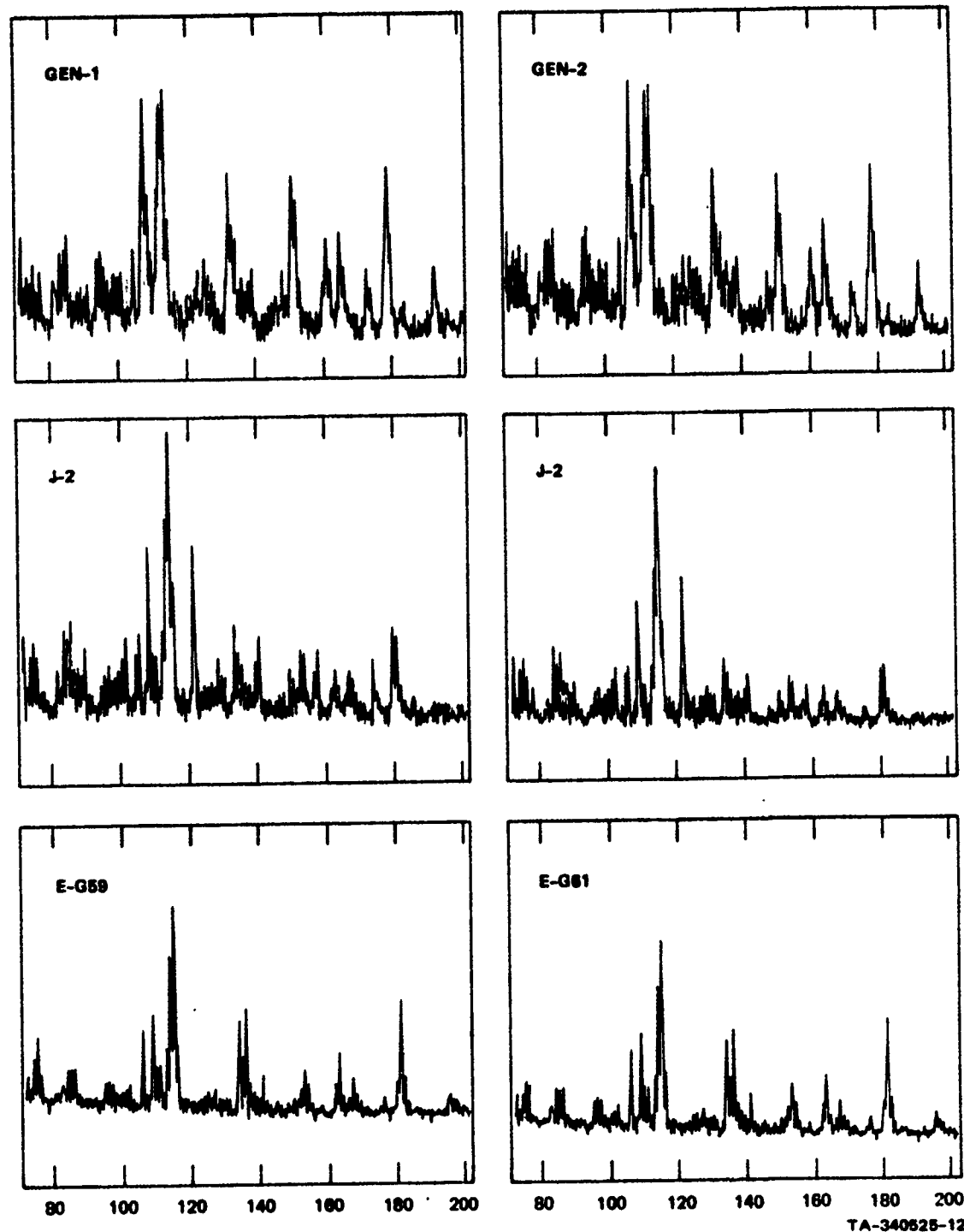


FIGURE 8 SIX MASS SPECTRA OF SAME INDIVIDUAL'S URINE SHOWING .
REPRODUCIBILITY OF METHOD

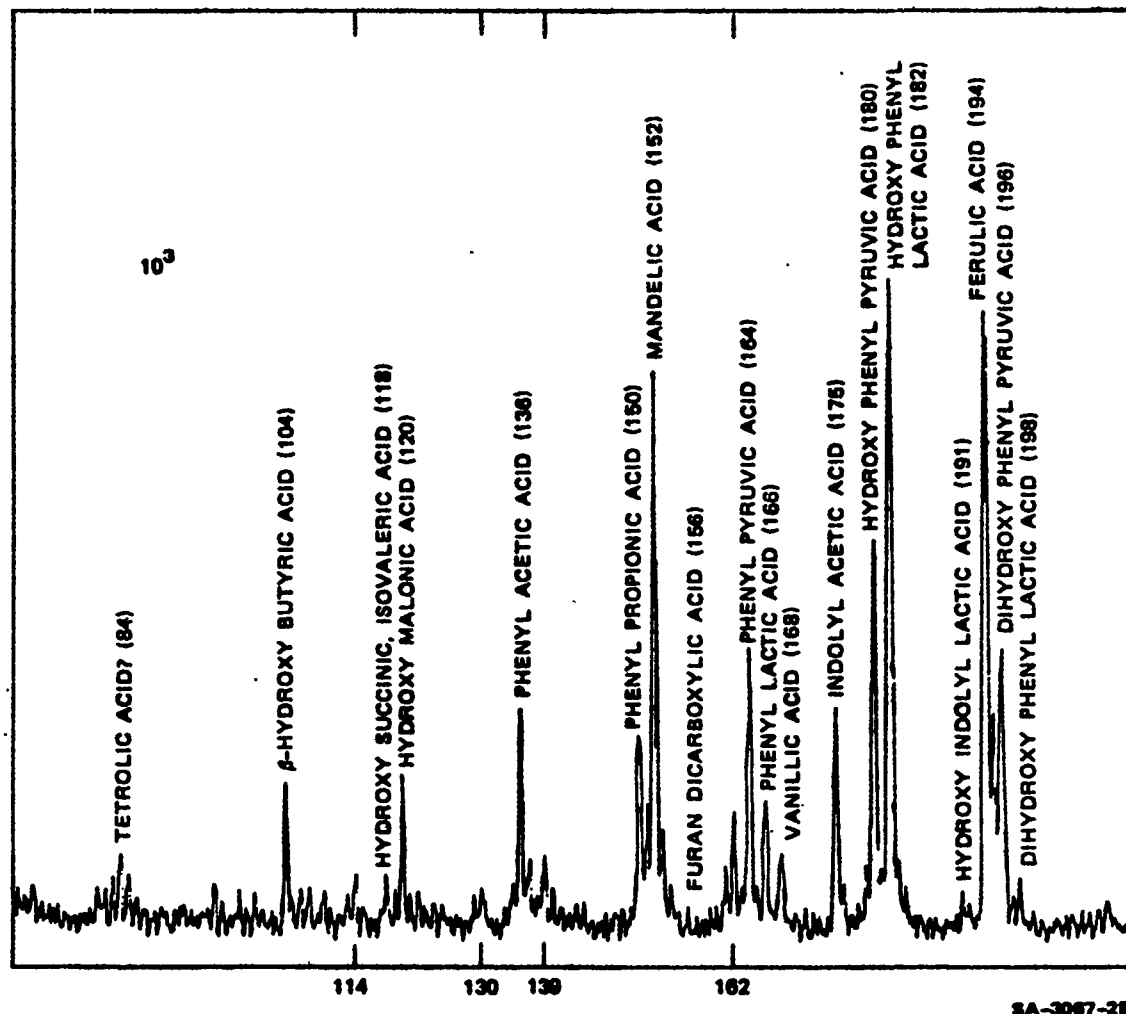


FIGURE 9 . CARBOXYLIC ACIDS EXTRACTED FROM NORMAL HUMAN URINE

the tlc³⁶ and glc⁵⁸ methods. Our methodology, unlike the previous ones, does offer a reproducible quantitative assay, however.

To check on possible losses of the more volatile carboxylic acids during the removal of the ethyl acetate under vacuum, we have modified the procedure as follows. Before evaporation, the solution was neutralized by the addition of a solution of NH_3 in ethyl acetate. Following complete evaporation, which now left the ammonium salts, the latter were dissolved in hexafluoroisopropanol. Dry HCl was then bubbled through this solution to release the carboxylic acids and the solution was transferred into the glass capillary containing glass powder and evaporated under dry N_2 at room temperature. The glass powder was used to reduce the possible evaporation of volatile carboxylic acids at the last stage of preparation. The mass spectrum obtained (Figure 10) does not differ significantly from that obtained without this special treatment.

To check on the reproducibility of spectra of carboxylic acids extracted from urine, four samples from the same urine were extracted identically and independently and then four aliquots of each sample were fingerprinted using field ionization mass spectrometry. Spectra were collected with a multichannel analyzer and stored on a 9-track magnetic tape. Six of these spectra are presented in Figure 11. A second urine from a different individual was prepared as above; however only 7 of the 16 spectra remained on the magnetic tape after an accidental erasure caused by an electrical arc in adjacent equipment. These 7 spectra were from two independent extractions--four from one and three from another.

An analysis of variance of the urine having all 16 spectra intact was undertaken by computer to check on the variability in sample preparation versus that occurring from run to run. From the analysis of variance table (Table 1), the variances S_e^2 , S_d^2 , and S_a^2 can be computed,

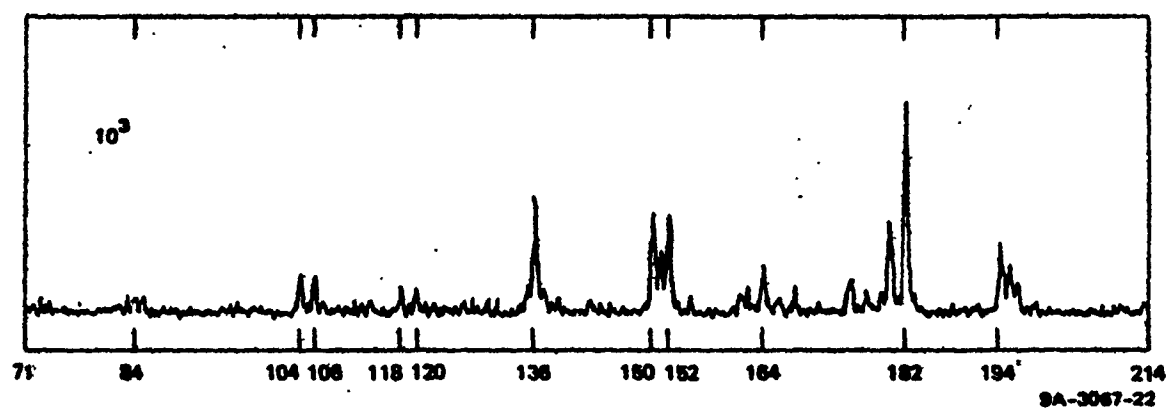
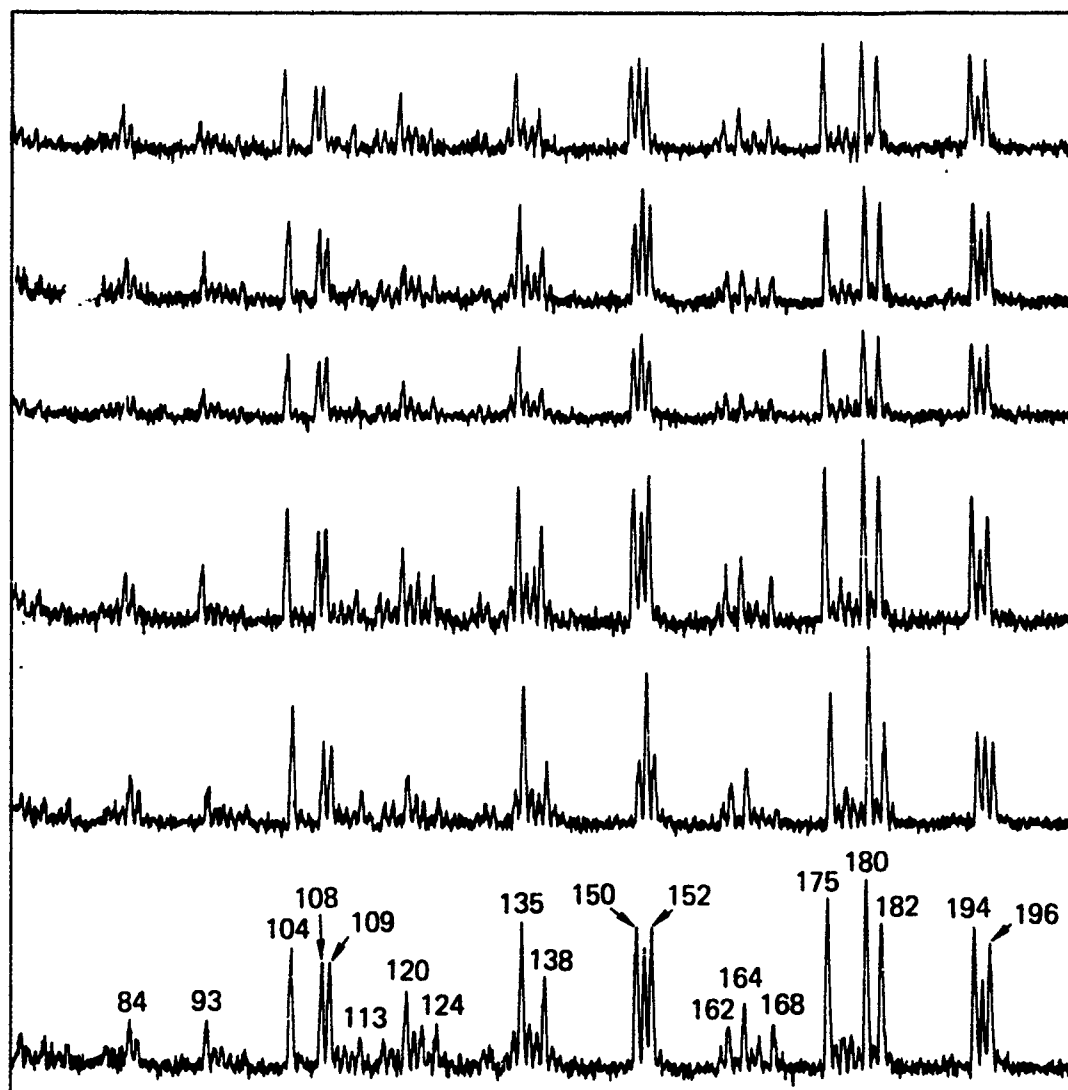


FIGURE 10 CARBOXYLIC ACIDS OF EXTRACTED URINE—AMMONIUM SALT PROCEDURE



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FIGURE 11 REPLICATION OF CARBOXYLIC ACID EXTRACTS FROM HUMAN URINE

where S_e^2 = sampling error variance, S_d^2 = variance caused by chemistry preparation, and S_a^2 = variance caused by instrumental variation from run to run.

Relative Standard Deviation %

$S_e^2 = 0.000972$ = sampling error	$S_e = 12.61$
$S_d^2 = 0.001275$ = chemistry prep.error	$S_d = 14.17$
$S_a^2 = 0.004588$ = instrumental error	$S_a = 26.88$

From the above, we conclude that instrumental error provides the largest source of variation in trying to reproduce a given carboxylic acid spectrum.

Table 1

ANALYSIS OF VARIANCE OF REPLICATE R-COOH SPECTRA

Source of Variation	Sums of Squares	Degree of Freedom	Mean Squares	Expected Mean Squares
Peaks	0.435	14	0.031	$\sigma^2 + 2\sigma_\delta^2 + 6\sigma_\epsilon^2$
Samples within Peaks	0.156	45	0.00347	$\sigma^2 + 2\sigma_\delta^2$
Observations within Samples	0.175	180	0.000972	σ^2
TOTAL	0.765	239		

The instrumental errors were more recently significantly reduced by improving the performance of the quadrupole mass spectrometer. The variance obtained at present is less than 3%.

Before field ionization mass spectrometric analysis of urine extracts from patients with known liver dysfunction, urines from normal hypo- and hyperactive patients were analyzed in collaboration with Dr. Meyer Friedman of Mt. Zion Hospital, San Francisco. More than 60 field ionization mass spectra were obtained of: carboxylic acid extracts of the urines collected on arising and at the end of the day from twenty of his patients, basic extracts of urines from ten of the patients; so as to determine if these spectra would be useful in classifying these patients as either hypo- or hyperactive. Statistical computer analysis did not demonstrate any significant difference between the two groups. The statistical method used to determine the differences between the two groups was described in an appendix submitted with our fifth quarterly report on this project (June 13, 1975). This result makes the difference observed recently in the case of the patients with infectious hepatitis even more significant from the diagnostic standpoint. As will be seen in Section F, the statistical analysis used in the latter case is also more rigorous than that used in this preliminary study.

We have also modified the extraction technique for carboxylic acids, substituting column chromatography on XAD-2 resin for the solvent extraction. The following new procedure has been used to analyze urines of patients with infectious hepatitis (obtained from Dr. Redecker of John Wesley County Hospital, Los Angeles):

Frozen urine specimens were thawed and shaken, and duplicate 1-ml aliquots were taken from each of 24 hepatitis patients. Twenty-four urine specimens from 14 volunteers at SRI were treated identically and served as controls. One milliliter of concentrated hydrochloric acid

was added as a safety factor to denature/deactivate any possible hepatitis virus. The mixture was neutralized to pH 7 with NaOH and run through a BioRad AG3-X4A anion exchange resin column (0.7 x 2 cm resin Bed) in the OH⁻ form, washed with 10 ml distilled water and followed by elution with 5 ml 0.5 N NaOH. This solution was then acidified to pH 3 with HCl and run through a column of Amberlite XAD-2 resin (0.7 x 4 cm resin bed), washed with 10 ml distilled H₂O and eluted with 5 ml absolute methanol. The organic solvent was removed under a nitrogen stream using a Meyer N-Evap analytical evaporator at 55°C. Samples were then redissolved in 10 drops of absolute MeOH and transferred to 1-ml glass culture tubes. Aliquots were further dried on the outside of a small glass capillary to undergo field ionization mass spectrometry analysis. The quality of spectra obtained is exemplified in Figures 12 and 13.

The preparation of neutral extracts from urine was also modified following the successful separation of the carboxylic acid. The new procedure is:

Urine specimens are detoxified with HCl as before, neutralized with NaOH and buffered with 4 ml 0.4 M phosphate buffer pH 6.8, treated with urease to remove urea, and brought up to pH 9 with 0.5 N NaOH. The samples are then passed over an XAD-2 column, washed with 10 ml distilled H₂O to remove salts and eluted with absolute MeOH and dried as before. The quality of spectra obtained is exemplified in Figure 14.

We are analyzing at present the neutral extracts from the same urines studied for their carboxylic acid content. The results will be presented in the annual report of the third phase.

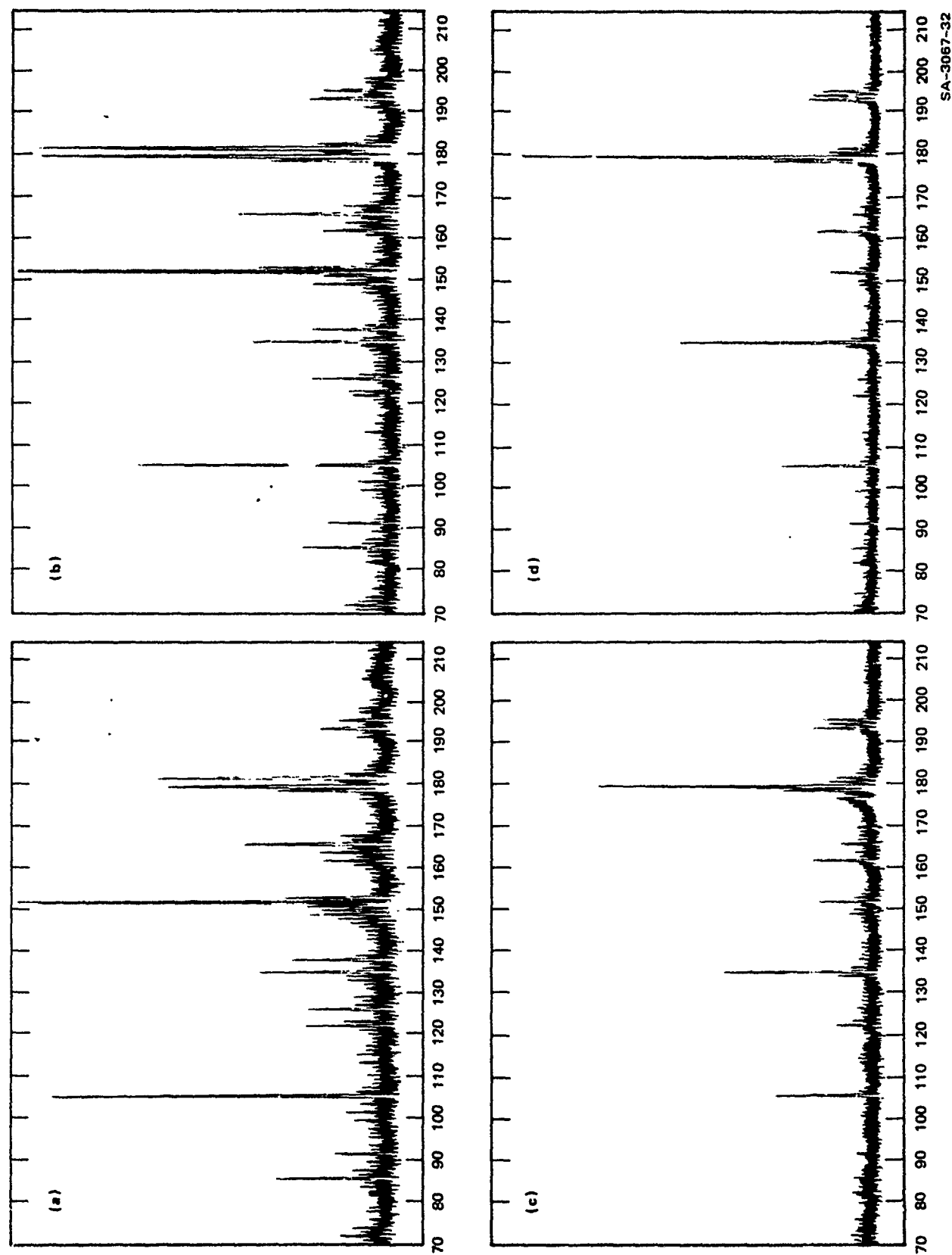
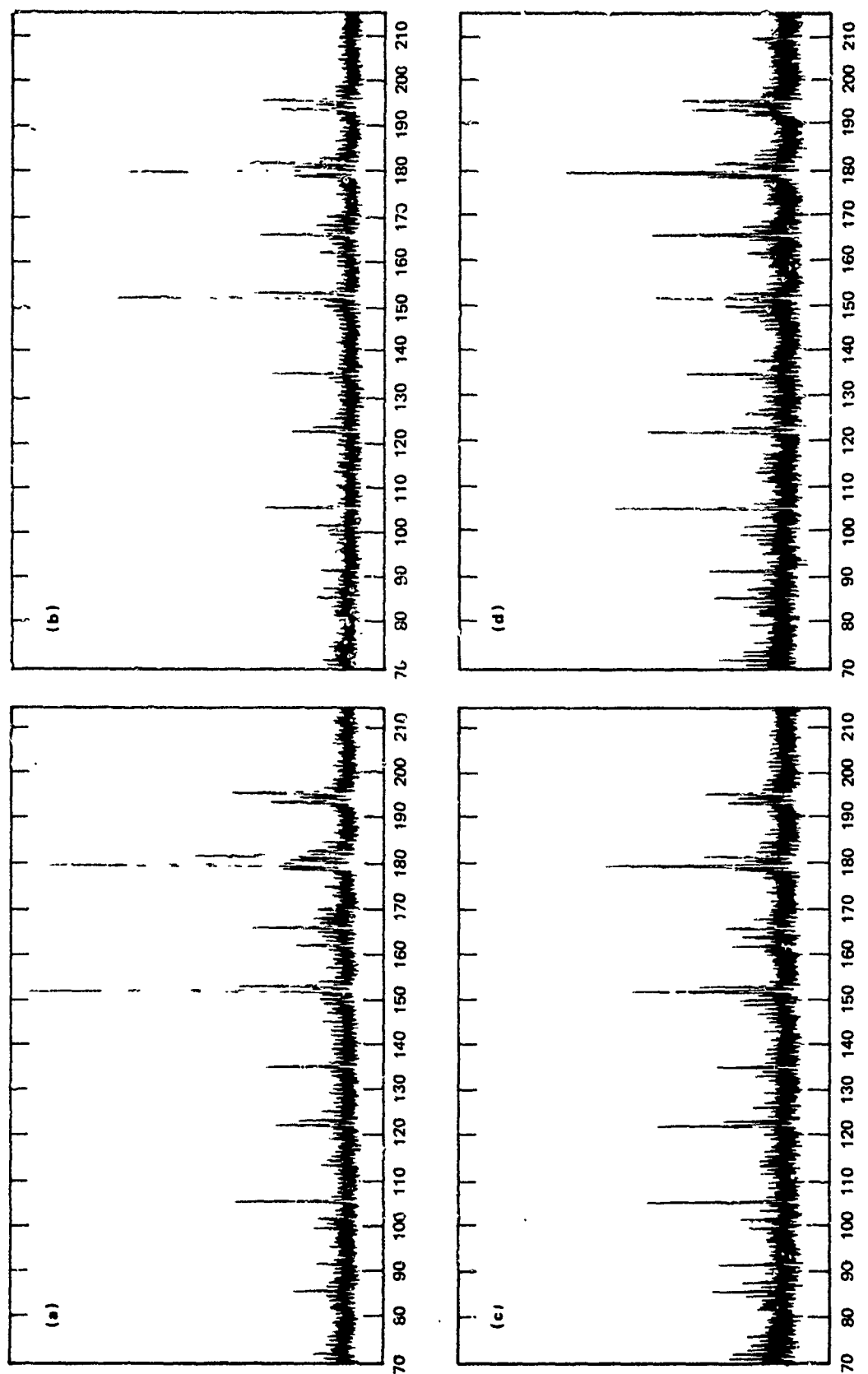


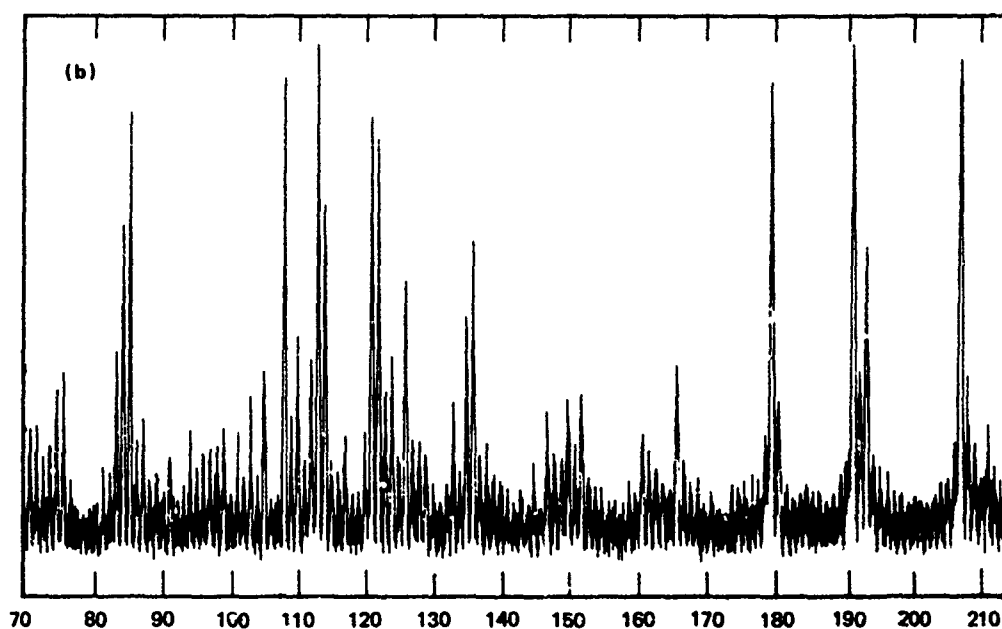
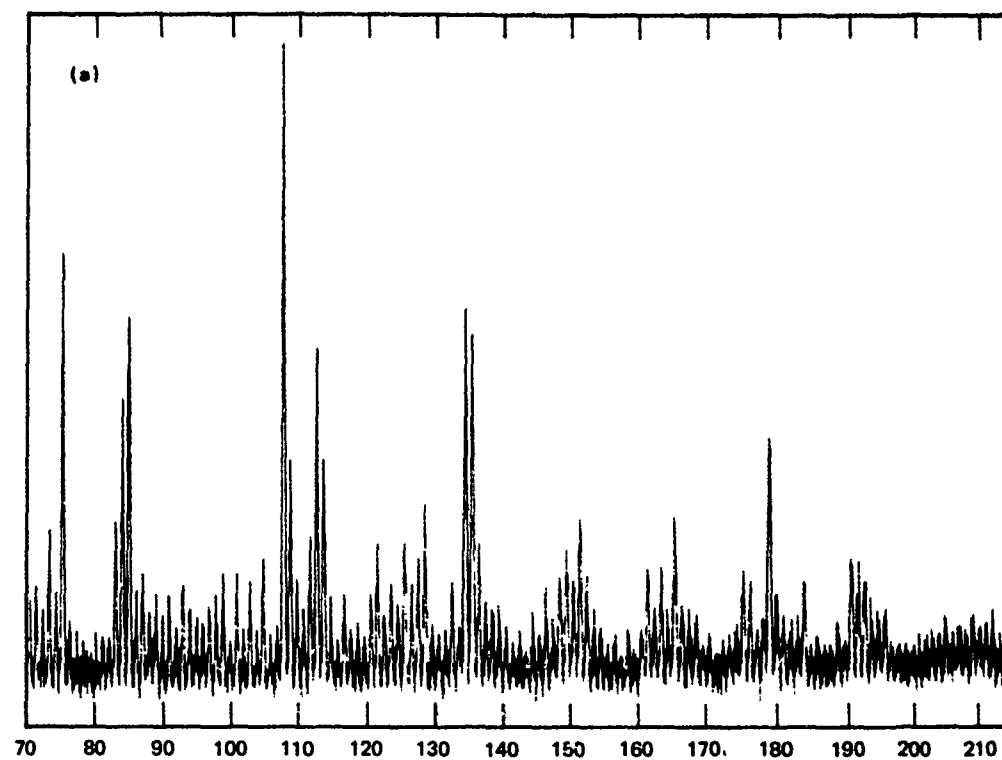
FIGURE 12 MOLECULAR WEIGHT PROFILES OF CARBOXYLIC ACIDS IN TWO NORMAL SUBJECTS

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FIGURE 13 MOLECULAR WEIGHT PROFILES OF CARBOXYLIC ACIDS IN TWO PATIENTS WITH INFECTIOUS HEPATITIS



SA-3067-34

FIGURE 14 NEUTRAL EXTRACT MOLECULAR WEIGHT PROFILE OF URINE FROM TWO NORMAL SUBJECTS

F. Statistical Analysis of Multicomponent Spectra

This section describes the computer programs used to process the raw data from the magnetic tape, followed by the statistical analysis for significant differences between normals and patients with infectious hepatitis. We have analyzed by field ionization mass spectrometry 48 urine samples in duplicate for the molecular weight profiles of their carboxylic acids. Half of these samples are from patients with infectious hepatitis.

The computer programs used for the data processing consisted of two groups. The first group processed the raw mass spectra obtained on the multichannel analyzer and recorded on magnetic tape. This was achieved after selecting the 39 most prominent peaks of the spectra, determining the area of each peak and normalizing it to the total area under these peaks. (The computer program flowsheet will be presented in the annual report).

The second group is of statistical analysis of the spectra. This analysis includes the Wilcoxon test for each of the 39 peaks followed by a second statistical test, the WNI test, on the diagnostic power of the methodology.⁶⁵

Let us describe now the steps of statistical analysis:

We have n_c spectra, representing n_c urine specimens from a control group (class C). Another n_p spectra represent a group of infectious hepatitis patients (class P).

To determine whether or not a difference exists between the two classes of spectra, we use the Wilcoxon test. The null hypothesis of this test is that both classes come from the same population (i.e., there is no difference between the two classes). By demonstrating that

the observed differences between the two classes of spectra are improbable under the null hypothesis, we conclude that the two classes do not come from the same population.

The Wilcoxon test is performed as follows: The data from both classes of normalized spectra are pooled into a single class of $N = n_c + n_p$ spectra. For each mass peak, we now have N numbers. Consecutive numbers are assigned ranks according to their magnitudes. The smallest area is assigned rank = 1, the second smallest area has a rank of 2 and so on until we reach rank N . The rank numbers are now separated into the original classes of their respective spectra and summed.

Let S_c and S_p denote the observed rank sums for the control and pathological groups respectively. Since $S_c + S_p = 1 + 2 + \dots + N$ (i.e., $S_c + S_p = \text{constant}$), the probability of obtaining any prescribed value of S_c is equal to the probability of obtaining its complement, S_p . Therefore, we will limit the following of the Wilcoxon test to S_c .

The average value of the ranks in the control group is $\bar{R}_c = S_c/n_c$. The expected average rank for the pooled group is given by

$$E[\bar{R}] = (1 + 2 + \dots + N)/N = (N + 1)/2$$

The variance in the mean rank for the control group is

$$\text{VAR}[\bar{R}_c] = \frac{(N + 1)n_c}{12(N - n_c)}$$

The variate $(\bar{R}_c - E[\bar{R}])/\sqrt{\text{VAR}[\bar{R}_c]}$ is an asymptotically unit normal variate. The integral

$$\Phi(u) = \frac{1}{\sqrt{2\pi}} \int_{-\infty}^u e^{-x^2/2} dx$$

is evaluated as a function of u in standard statistics tables. For the case, $u = (S_c/n_c - E[\bar{R}])/\sqrt{\text{VAR}[\bar{R}_c]}$, $p = 1 - \Phi(u)$ is the probability that

a rank sum as extreme (large or small) or more than the observed value S_c could be obtained under the hypothesis that the rank numbers were picked at random from the same population. In other words the value of p is a measure of how likely it is to obtain the value S_c if the two groups were indistinguishable in terms of the j^{th} peak. Therefore, small values of p ($p \leq 0.1$, for example) imply that the observed value S_c is improbable under the null hypothesis and we can conclude that the two groups are distinguishable in terms of the j^{th} peak. When several peaks have small "p-values", we can conclude that a pattern difference exists between the two groups of spectra.

The Wilcoxon test, described above, makes no assumptions about the way in which the peak areas are distributed about their mean values (i.e., it is a nonparametric test). Figure 15 shows the number of peaks with p values $\leq p$ versus p (i.e., a cumulative distribution). As can be seen in the figure, more than half of the peaks have p values less than 0.05 and all the peaks have p values less than 0.5. In other words, the observed differences in rank sum are not very probable under the null hypothesis--hence we conclude that the spectra are from different populations with distinguishable pattern differences.

The areas of the 39 peaks of each of the 24 duplicate spectra of the normal and of the hepatitic spectra have been averaged and normalized to give two composite spectra with values of $\bar{A}_j(C)$ and $\bar{A}_j(P)$ for the areas of each of the peaks. The values of $\bar{A}_j(C)$ and $\bar{A}_j(P)$ as well as of the p values of each of the peaks are presented in Table 2. This table identifies each of these peaks by its mass number. The two composite spectra as well as the "spectrum" of the p values are presented in Figure 16. It is evident that the two composite spectra differ significantly from each other and the "spectrum" of the p values identifies again the constituents of highest diagnostic value. This presentation

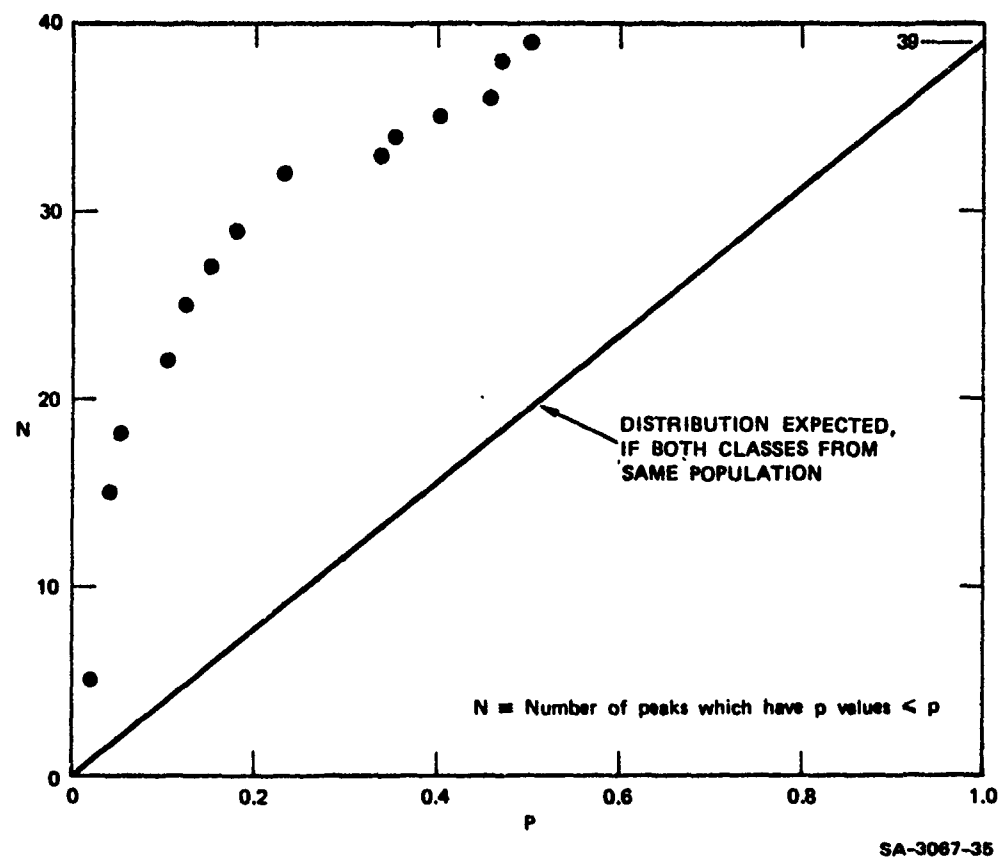
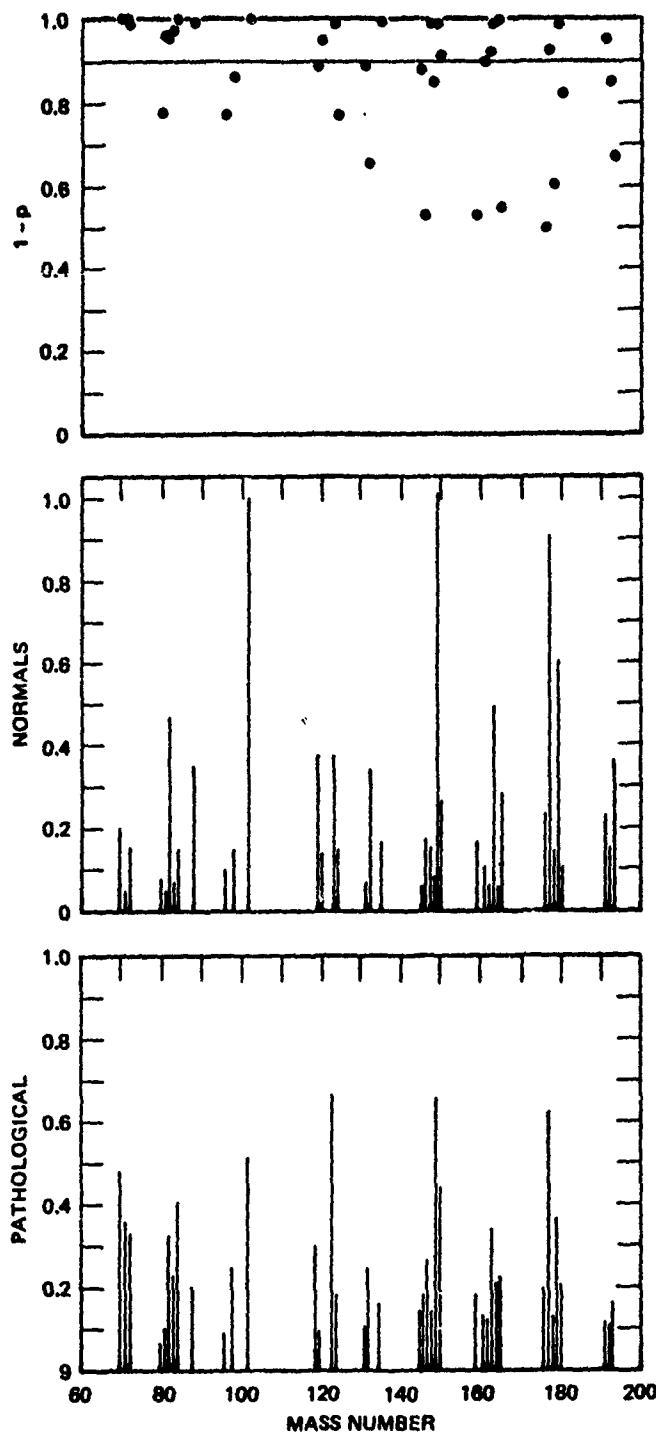


FIGURE 15 CUMULATIVE DISTRIBUTION OF $N(P)$



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FIGURE 16 COMPOSITE SPECTRA OF NORMAL AND HEPATITIC URINES

Table 2

J	M amu	\bar{A}_j (C)	\bar{A}_j (P)	p
1	70	0.0201	0.0475	0
2	71	0.0050	0.0358	0.0001
3	72	0.0152	0.0326	0.0139
4	80	0.0080	0.0062	0.2236
5	81	0.0051	0.0100	0.0376
6	82	0.0469	0.0327	0.0455
7	83	0.0071	0.0229	0.0228
8	84	0.0150	0.0406	0
9	88	0.0350	0.0200	0.0091
10	96	0.0104	0.0083	0.2266
11	98	0.0150	0.0243	0.1357
12	102	0.1000	0.0514	0.0006
13	119	0.0373	0.0300	0.1160
14	120	0.0136	0.0094	0.0495
15	123	0.0377	0.0665	0.0071
16	124	0.0144	0.0186	0.2296
17	131	0.0071	0.0107	0.1112
18	132	0.0345	0.0245	0.3470
19	135	0.0167	0.0161	0.0018
20	145	0.0057	0.0142	0.1190
21	146	0.0176	0.0183	0.4680
22	147	0.0155	0.0268	0.0034
23	148	0.0081	0.0144	0.1539
24	149	0.1008	0.0665	0.0071
25	150	0.0265	0.0342	0.0823

Table 2 (cont'd)

J	M amu	\bar{A}_j (C)	\bar{A}_j (P)	p
26	159	0.0166	0.0186	0.4721
27	161	0.0107	0.0136	0.0968
28	162	0.0061	0.0123	0.0778
29	163	0.0497	0.0341	0.0075
30	164	0.0056	0.0211	0.0034
31	165	0.0283	0.0229	0.4540
32	176	0.0232	0.0200	0.4960
33	177	0.0904	0.0630	0.0749
34	178	0.0144	0.0134	0.4010
35	179	0.0607	0.0371	0.0140
36	180	0.0104	0.0211	0.1780
37	191	0.0230	0.0121	0.0465
38	192	0.0154	0.0116	0.1492
39	193	0.0269	0.0167	0.3336

reiterates the presentation of Figure 15 that 22 of the 39 constituents of the carboxylic acids (of molecular weights 70 to 193) excreted in urine have diagnostic value and that 11 out of 39 peaks have a diagnostic significance of >99%, but Figure 16 identifies their constituents by their molecular weights.

When our fragmentation chamber mass spectrometer, referred to in Section C, will become operational we may be in a position to determine the chemical identity of each of these critical constituents. The fact that as many as 22 out of 39 peaks have significant diagnostic value should not surprise us in view of the fact that the disease under consideration is a liver disorder which is expected to alter significantly the metabolic fate of many metabolites.

The computation of p-values was performed as a measure of the pattern difference between the control and pathological spectra. To evaluate the significance of this pattern difference in terms of its diagnostic merit we computed weighted noncorrelation indices, WNI, for each spectrum. The WNI for comparing the i^{th} spectrum to the average control spectrum is defined as

$$WNI_i(C) = 100 \sum_{j=1}^{39} \frac{A_{ij} - \bar{A}_j(C) r_j}{A_{ij} + \bar{A}_j(C)} \bigg/ \sum_{j=1}^{39} r_j ,$$

where A_{ij} is the normalized area of the j^{th} peak of the i^{th} spectrum, $\bar{A}_j(C)$ is the j^{th} average peak of the control group and r_j is a weighting factor. For peaks which had p values < 0.01 a weight of 1.0 was assigned to r_j , otherwise $r_j = 0$. The $WNI_i(C)$ is a measure of how closely an individual spectrum matches the average spectrum of the control group. If we replace $\bar{A}_{ij}(C)$ by $\bar{A}_{ij}(P)$, we obtain the $WNI_i(P)$ for comparing the spectra to the average pathological spectrum. For example, if $r_j = 1$ for all 39 peaks, the $WNI(C)$ equals the average absolute fractional deviation (expressed in percent) of an individual spectrum from the

average control spectrum. The effect of the weighting factors is to disregard the peaks with large p-values.

In order for the pattern differences to be useful as a diagnostic tool, we need the following conditions:

classification of i^{th} spectrum	classification of \bar{A}_j	size of WNI
C	C	small
P	P	small
C	P	large
P	C	large

When these conditions are met, the difference,

$$D_i = \text{WNI}_i(\text{C}) - \text{WNI}_i(\text{P})$$

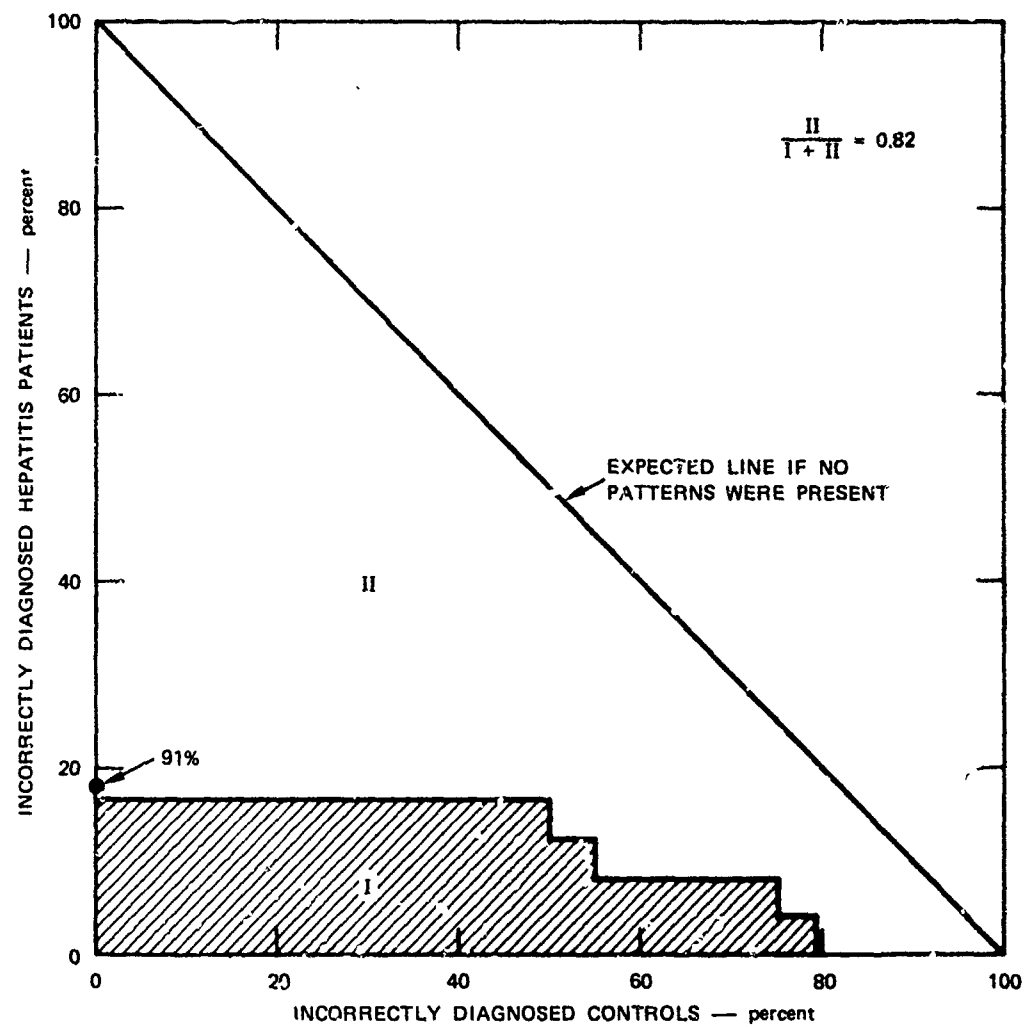
will be positive when the i^{th} spectrum represents a pathological case and negative when it represents a control case.

The differences D_i were computed for each of the control and pathological spectra. D_i represents a possible diagnostic criterion. If we take each of the observed D_i and use it as a level above which (i.e., more positive than) the diagnostic decision is "pathological" and below which it is "non-pathological", we can compute the percentage of correct and incorrect diagnoses that would have been performed on the present data as a function of D_i . This computation was performed for all of the D_i 's. For the case $D_i = 0$, the percent correct diagnoses averaged over pathological and control spectra was 91%. It should be remembered that this calculation was made on the basis of just 24 pathological cases versus 24 normals. In the long run a diagnostic test would be based on a minimum of 50 cases of each type which would certainly

reduce the variances of $\bar{A}_j(C)$ and $\bar{A}_j(P)$ and therefore increase the reliability of diagnosis. What our present results mean is that when we try to diagnose the subjects under consideration, we find 24 out of 24 healthy subjects as healthy, 22 out of the 24 hepatic patients as sick and we miss only on two patients. Even these two subjects may belong to a different population and might have been diagnosed wrongly in the hospital or may have already recovered from the acute stage of the disease.

Another figure of merit is the diagnostic power. In Figure 17 the percent incorrectly diagnosed pathological cases is plotted versus the percent incorrectly diagnosed control cases. The diagnostic power computed from this graph is $DP = 0.82$, which compares very favorably to the DP's of 0.71 and 0.75 obtained by Robinson and Westall for the diagnosis of multiple sclerosis through amino acid analysis using ion exchange chromatographic techniques.

In the next two months we hope to complete the analysis of the same 48 samples of urine for their nonacidic metabolites and compare the diagnostic power of the two groups of metabolites. These results, as well as further details on our computation techniques will be presented in the report on the third phase of this program.



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FIGURE 17 DIAGNOSTIC POWER OF TECHNIQUE

IV PROPOSED PROGRAM OF RESEARCH FOR THIRD PHASE

In the proposed third phase of this program, we intend to accomplish the following tasks:

- (1-a) Substantiate the diagnostic test for infectious hepatitis by analyzing more urine samples from patients, after deciding whether to use carboxylic acids or the neutral extracts as the diagnostic parameter.
- (1-b) Extend the diagnostic tests to urines from subjects who recovered from infectious hepatitis to determine whether the characteristic pattern of metabolites is associated with the acute phase of the disease, or is an indication of a more or less persistent liver damage. Also, we hope to ascertain whether this test could detect cases of non-symptomatic carriers.
- (1-c) Examine urine of patients with liver diseases other than infectious hepatitis to find out whether the pathological pattern demonstrated by us is indicative of general liver damage or of the viral infection.
- (2) Establish the identity of individual metabolites whose concentrations show the largest differences between normals and patients with infectious hepatitis. This could enable us to identify the biochemical nature of the pathological aberration.
- (3) Examine the possibility of limiting the multicomponent analysis to the ten components which exhibit the most significant diagnostic value. Such a procedure could cut down the time of analysis and simplify the computational process. Using the new rapidly recyclable field ionization source and a dedicated minicomputer (or a mass preselector) interfaced with the quadrupole analyzer, we could mass-analyze ten to twelve samples per hour. The newly developed sample preparation procedure allows the processing of up to 24 samples per man-day. Thus, a clinical laboratory could achieve a routine throughput rate of 50 to 100 multicomponent analyses of urine in a two-shift day operations of a single mass spectrometer.

- (4) Analyze bacterial extracts by the new procedure developed for neutral constituents of urine. This procedure seems to be faster and more reproducible than the previously used solvent extraction. The instrumentation and computerized analysis developed during the second phase of this project should enable rapid progress on the original objective of identifying microorganisms by multicomponent analysis.

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